

# First record of *Stylostomum ellipse* (Dalyell, 1853) (Platyhelminthes, Polycladida) from the Pacific Ocean

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## Abstract

The polyclad flatworm *Stylostomum ellipse* (Dalyell, 1853) has hitherto been recorded from the Antarctic region, Mediterranean Sea, Patagonian region, Scandinavia, South Africa, and South Georgia Island. In this study, we report *S. ellipse* for the first time from the Pacific Ocean based on specimens collected in Hokkaido, northern Japan. Our specimens are morphologically identifiable as *S. ellipse*, but may represent a biologically different species from a population of the Mediterranean Sea. This is because, based on the previous genetic data of other cotylean species, the observed uncorrected *p*-distance 0.02160 between the two distinct populations in terms of a partial 972 bp region of the 28S rDNA sequence may be great enough to separate the species biologically.

## Keywords

Cosmopolitan species, Euryleptidae, genetic distance, marine invertebrates, taxonomy.

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## Introduction

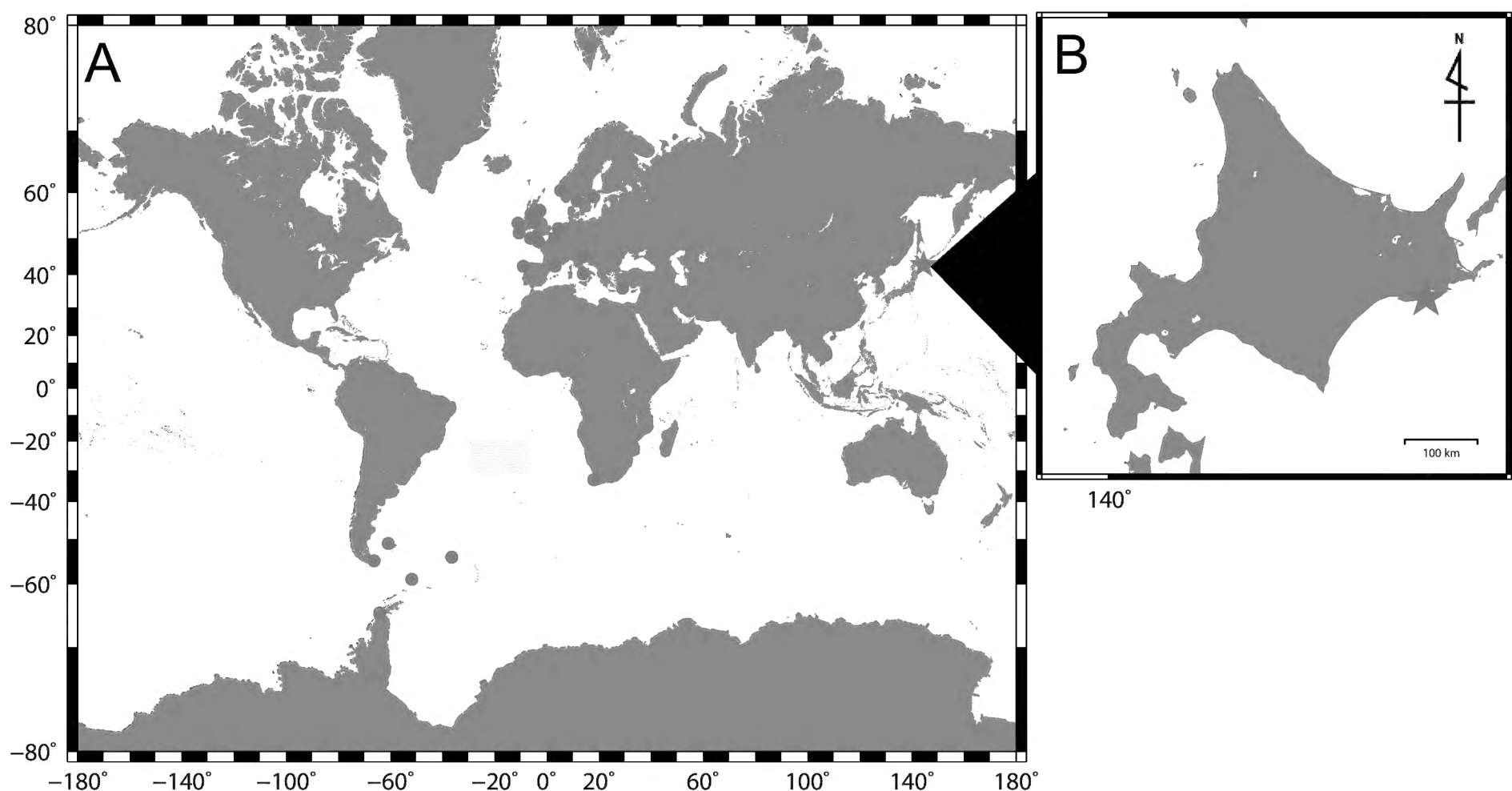
The euryleptid flatworm genus *Stylostomum* Lang, 1884 sensu Holleman (2001) is characterized by the oval body shape and the small to moderate size; the mouth and the male gonopore in common opening to the exterior; and the tentacles reduced to small stumps or lacked. *Stylostomum* consists of nine valid species: *S. ellipse* (Dalyell, 1853); *S. felinum* Marcus, 1954; *S. frigidum* Bock, 1931; *S. hozawai* Kato, 1939; *S. lentum* Heath & McGregor, 1912; *S. maculatum* Kato, 1944; *S. mixtomaculatum* Pitale & Apte, 2019; *S. sanjuania* Holleman, 1972; and *S. spanis* Holleman, 2001. All are distributed in the boreal and temperate realms north of latitude 36°N and south of latitude 36°S (Holleman 2001), except *S.*

*mixtomaculatum*, recorded from the west coast of India (Pitale and Apte 2019).

*Stylostomum ellipse* is known to have the most discontinuous distribution among the polyclads (Prudhoe 1985). This species has been recorded in both the north-eastern and southern parts of the Atlantic: Scandinavia to Mediterranean Sea, South Africa, the Patagonian region, South Georgia Island, and the Antarctic region (Fig. 1A). In this paper, we present the first record of *S. ellipse* from the Pacific Ocean.

## Methods

Eight polyclad specimens were collected subtidally by dredging in the Pacific coast of Hokkaido, northern



**Figure 1.** Distribution of *Stylostomum ellipse*. Stars indicate the new record from Hokkaido, northern Japan. Circles indicate the previously known records around the Atlantic Ocean. **A.** Updated distribution map. **B.** Magnification of Hokkaido, showing the sampling site of this study.

Japan (Fig. 1B). Morphological preparation and molecular methods are mostly in accordance with those in Tsuyuki et al. (2019). Worms were anesthetized in a  $MgCl_2$  solution prepared with tap water so that it had the same refractive index (or “salinity”) as the seawater, using an IS/Mill-E refractometer (AS ONE, Japan), and then photographed with a Nikon D5300 digital camera with external strobe lightning provided by a pair of Morris Hikaru Komachi Di flash units. For DNA extraction, a posterior fragment of the body was removed and stored in 99.5% ethanol. The rest of the body was fixed in Bouin’s solution for 24 h and preserved in 70% ethanol for long term storage.

For histological examination, tissues were dehydrated in an ethanol series, cleared in xylene, embedded in paraffin wax, and sectioned at a thickness of 7  $\mu m$  using a microtome. Sections were stained with hematoxylin and eosin, mounted on glass slides in Entellan New (Merck, Germany), and then observed and photographed under an Olympus BX51 compound microscope. All vouchers are deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan.

Total DNA of six specimens was extracted using a silica-based method (Boom et al. 1990) after specimens were homogenized. A 979-bp fragment of the 28S rDNA was amplified with the primer pair fw1 and rev2 (Sonnenberg et al. 2007). Polymerase chain reaction (PCR) amplification conditions were: 94 °C for 30 s, 52.5 °C for 30 s, and 72 °C for 2 min; for 35 cycles in PCR amplification. All nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit ver. 3.1 and ABI 3730 Genetic Analyzer (Life Technologies, California, USA); and two internal primers, fw2 and rev4 (Sonnenberg et al. 2007), were used in addition

to fw1 and rev2. Sequences were checked and edited by using MEGA ver. 7.0 (Kumar et al. 2016). The edited sequences are deposited in DDBJ/EMBL/GenBank. The 28S rDNA uncorrected *p*-distance was calculated using MEGA ver. 7.0 (Kumar et al. 2016).

All graphical treatments were done with Adobe Photoshop CC 2019. Illustrations were prepared with Adobe Illustrator CC 2019.

## Results

Family Euryleptidae

Genus *Stylostomum* Lang, 1884

### *Stylostomum ellipse* (Dalyell, 1853)

Figures 2–4

*Planaria ellipsis* Dalyell 1853: 101–102, pl. 14, figs 9–16 (Scotland).

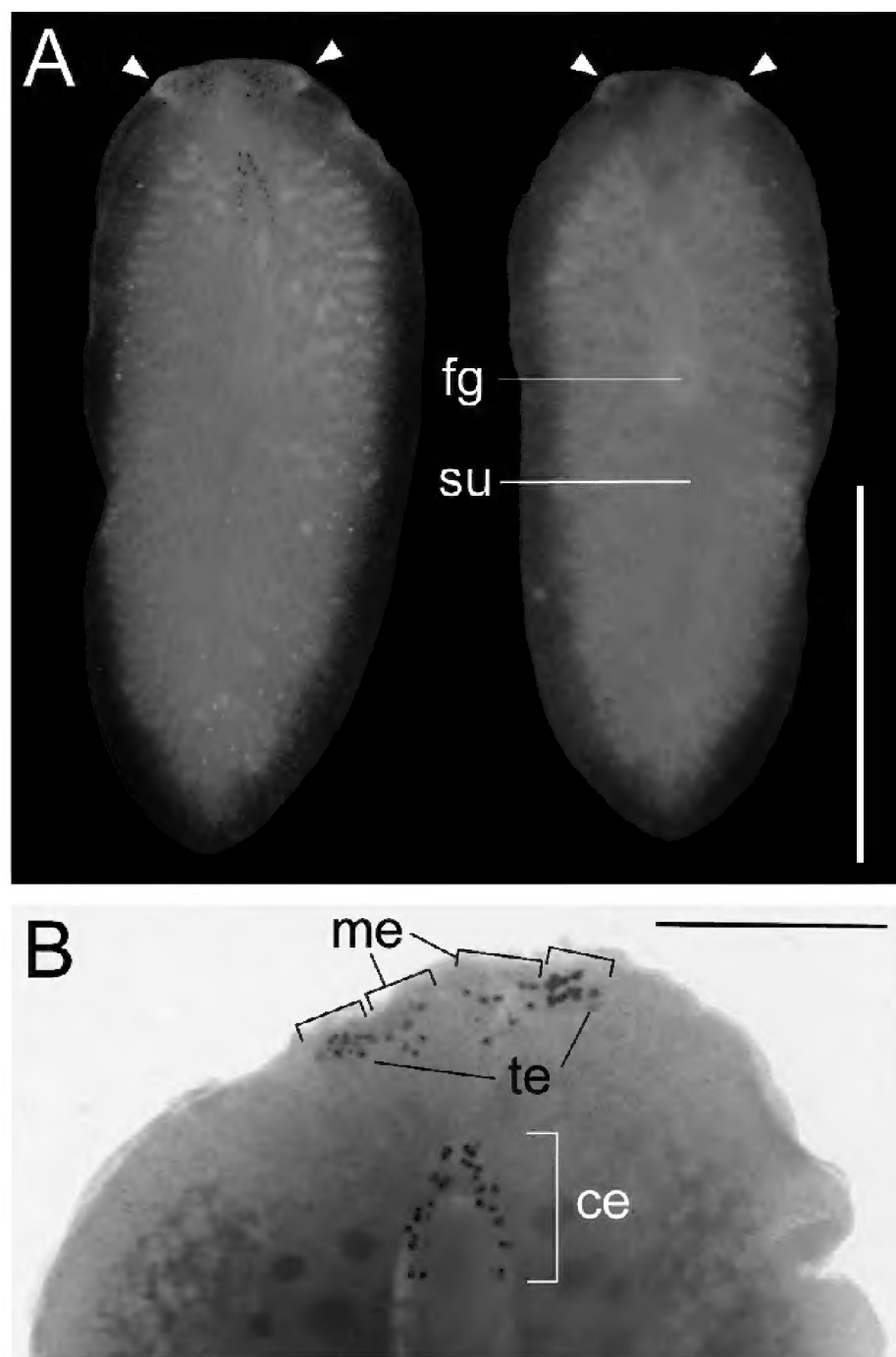
*Polycelis ellipsis*—Leuckart (1859: 183).

*Leptoplana ellipsis*—Diesing (1862: 542), Johnston (1865: 7), McIntosh (1874: 150), McIntosh (1875: 107).

*Stylochus roseus* Sars in Jensen 1878: 75, pl. 8, figs 1–3 (Norway).

*Stylostomum ellipse*—Lang (1884: 588), Gamble (1893a: 511), Bock (1913: 270–273; Koster, Sweden; Spitzbergen, Norway; Falkland Islands; Tierra del Fuego; South Africa), Steinböck (1932: 334, 337), Steinböck (1933: 20; Croatia), Bresslau (1928–1933: 241, fig. 240), Stummer-Traunfels (1933: 3575, fig. 154), Westblad (1952: 9–10; Falkland Islands; South Georgia; Tierra del Fuego), Crothers (1966: 22; Wales), Laverack and Blackler (1974: 32), Hendelberg (1974: 15, 17, figs 22–24; Sweden), Galleni and Puccinelli (1981: 42; Britain), Prudhoe (1982: 68–69, figs 24A, B; Scotland), Faubel (1984: 223), Prudhoe (1985: 141), Holleman (2001: 227–229), Faubel and Warwick (2005: 23–25, fig 6; off the Day Mark), Faasse and Ligthart (2007: 44–46, figs 1, 2; Netherlands); Cinar (2014: 707, 718; Turkey), Noreña et al. (2014: 18, fig 7C; Iberian Peninsula), Dittmann et al. (2019: 588, table 1; Croatia).

*Stylostomum roseum*—Lang (1884: 589), Bock (1913: 270).



**Figure 2.** *Stylostomum ellipse*, ICHUM 6003, from Japan. **A.** Entire animal in anesthetized living state, dorsal (left) and ventral (right) views; marginal tentacles indicated by arrowheads. **B.** Magnification of the same body in fixed state after being cleared in xylen; dorsal view, showing position and arrangement of cerebral and marginal eyespots. Abbreviations: ce, cerebral eyespots; fg, female gonopore; me, marginal eyespots; su, sucker; te, tentacular eyespots. Scale bars: 5 mm (**A**); 1 mm (**B**).

*Stylostomum variable* Lang 1884: 73, 585–588, pl. 8, figs 3, 4, 6, pl. 30, fig. 14, pl. 36, fig. 22 (Gulf of Naples); Carus (1885: 157), Lo Bianco (1888: 400); Vaillant (1889: 656; France), Bergendal (1890: 327; Sweden); Gamble (1893a: 511–513, pl. XXXIX, fig. 1; Isle of Man), Gamble (1893b: 47; England), Gamble (1893c: 171, pl. XIV, figs 43–46; Isle of Man), Hallez (1893: 178–180, pl. 3, fig. 9, pl. 4, figs 9–11), Hallez (1894: 230–233, pl. 2, figs 9–11), Plehn (1896: 172; Patagonia), Browne et al. (1898: 813; Ireland), Micoletzky (1910: 180; Trieste), Bock (1913: 270), Southern in Farran (1915: 36; Ireland), Southern (1936: 72; Ireland), Bassindale and Barrett (1957: 251; Stockholm), Eales (1961: 51).

*Stylostomum sanguineum* Hallez 1893: 180, 197, pl. 3, fig. 10, pl. 4, figs 12–14 (southern France); Hallez (1894: 233–235, pl. 2, figs 12–14; France), Bock (1913: 270, 273).

?*Stylostomum antarcticum* Hallez 1905: 126 (Antarctica); Hallez (1907: 10–11, pl. 1, fig. 6, pl. 2, fig. 5, 6).

?*Stylostomum punctatum* Hallez 1905: 126–127 (Antarctica); Hallez (1907: 10, pl. 2, fig. 1–4).

**Material examined.** JAPAN • 8 specimens; Hokkaido, Akkeshi Bay, Nakanose, Research and Training Vessel *Misago-maru* (Akkeshi Marine Station, Hokkaido University); 43°00.36'N, 144°46.86'E; depth 6.4–8.4 m; 25 June 2017; Y. Oya leg.; ICHUM 6000. • 3 specimens; same data as above, except 28 June 2018; ICHUM

6001–6003. • 4 specimens; same data as above, except 20 June 2019; ICHUM 6004–6007.

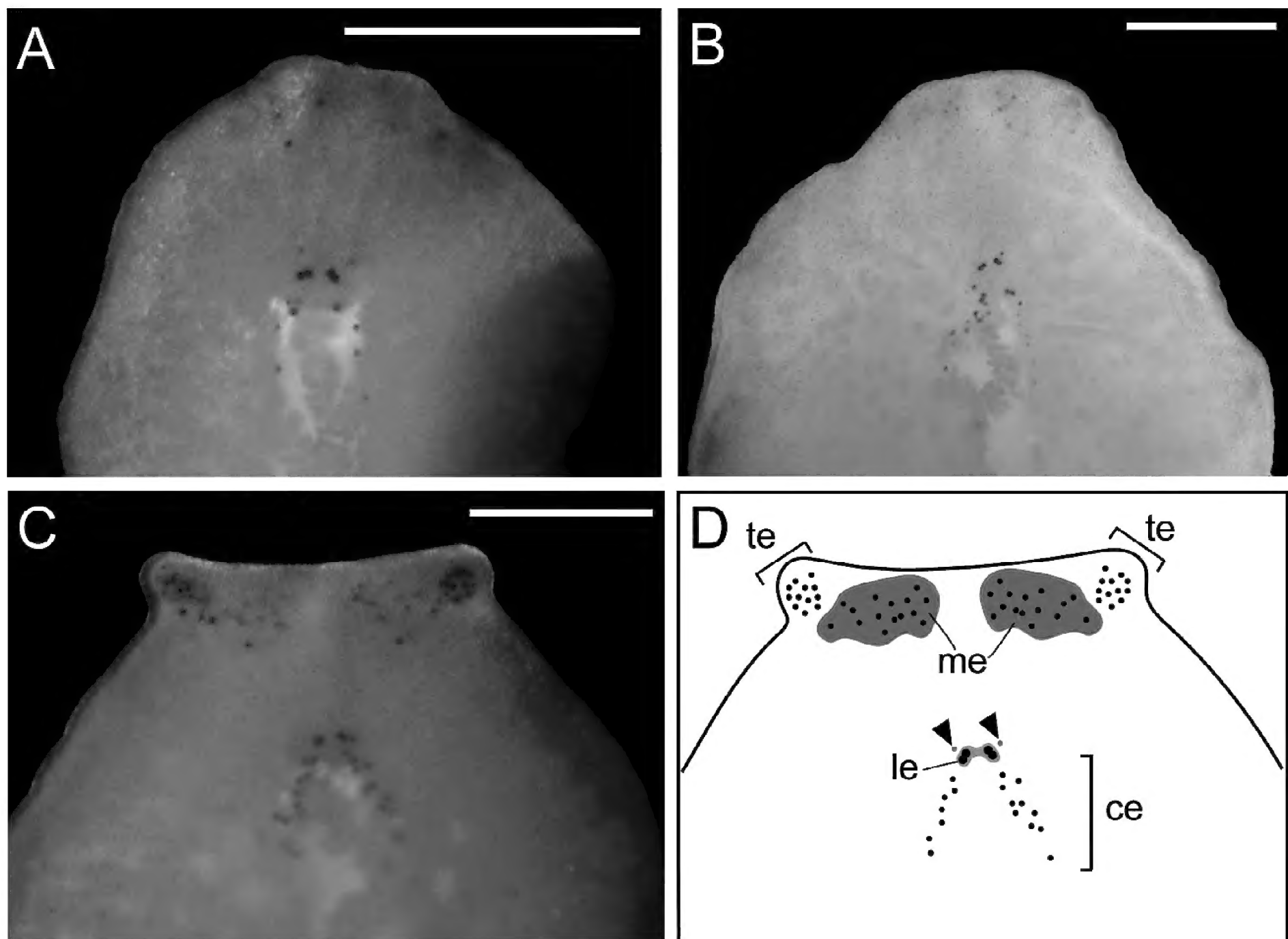
**Identification.** Body oval, 4.0–12.3 mm in length, 1.8–5.8 mm in maximum width in living state. Marginal tentacles rudimentary. Dorsal surface smooth, translucent, variably colored (white, orange, or red) depending on gut contents (Figs 2A, 3A–C). Body margin transparent. Intestine highly branched, spreading all over body; median intestinal branches absent in front of pharyngeal pouch. Pair of cerebral-eyespot clusters extending from brain to middle portion of pharyngeal pouch; each cluster consisting of 4–22 eyespots; two large cerebral eyespots, closely set to each other, located at anterior-most region in each cluster (Fig. 2B); additional, single, small cerebral eyespot embedded in parenchyma located closely anterior to large cerebral eyespot on each side (Fig. 3D, indicated by arrowheads), only found in ICHUM 6001, 6003–6006 (Fig. 3A, B). Marginal and tentacular eyespots spreading around two marginal tentacles (Fig. 2B). Ventral eyespots absent. Plicated pharynx tubular in shape. Male atrium and mouth sharing common pore, opening in front of pharynx (Fig. 4A, B). Male copulatory apparatus consisting of large seminal vesicle, spherical prostatic vesicle, and armed penis papilla, located under pharynx (Fig. 4C). Spermiducal vesicles well developed, forming single row on each side of midline, separately entering into seminal vesicle. Seminal vesicle oval, coated with thick muscular wall. Prostatic vesicle half as large as seminal vesicle, former situated above latter (Fig. 4A, C). Penis papilla armed with pointed tubular stylet, enclosed in penis pouch, protruding into male atrium. Female reproductive system posterior to pharynx. Female gonopore and sucker situated posterior to the male atrium–mouth common pore (Fig. 4A, D, E). Uterus bending directly downward, splitting into left and right immediately behind cement pouch (Fig. 4D). Uterine vesicle developed when containing eggs. Cement glands numerous, concentrated around vagina and releasing their contents into cement pouch.

**Sequences.** Partial 28S rDNA sequences (979 bp) from four individuals: LC508269 from ICHUM 6002; LC508270 from ICHUM 6003; LC508271 from ICHUM 6004; and LC508272 from ICHUM 6006. All were completely identical.

**Genetic distance.** In terms of partial 28S rDNA sequences, the uncorrected *p*-distance between the overlapping 972 bp of LC508269–508272 (979 bp) from Japan, and GenBank MN384704 (1395 bp) from Punat, Adriatic Sea (Dittmann et al. 2019), was 0.02160.

**Remarks.** The following morphological characteristics observed in our specimens correspond to the diagnostic characteristics of *S. ellipse* provided by previous researchers (Lang 1884; Bock 1913; Marcus 1954): a pair of two large cerebral eyespots anterior to smaller ones in each cerebral-eyespot cluster, and seminal vesicle twice as large as the prostatic vesicle.





**Figure 3.** *Stylostomum ellipse*, variations of the arrangement of eyespots. **A.** ICHUM 6006. **B.** ICHUM 6001. **C.** ICHUM 6007. **D.** Distribution of eyespots, showing positions of small cerebral eyespots embedded in parenchyma (indicated by arrowheads). Abbreviations: ce, cerebral eyespots; le, pair of two large cerebral eyespots; me, marginal eyespots; te, tentacular eyespots. Scale bars: 500  $\mu$ m (**A–C**).

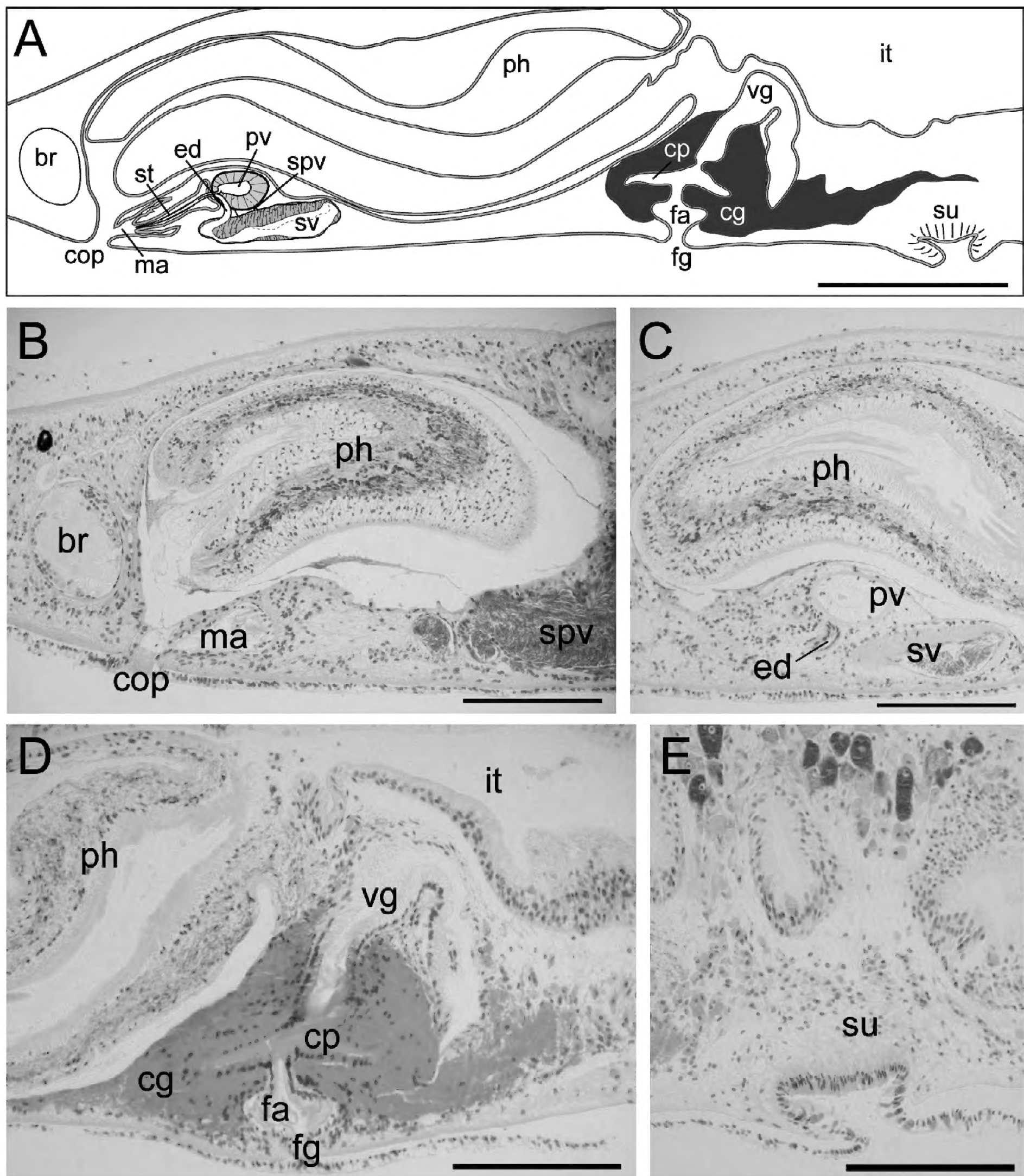
*Stylostomum ellipse* has potentially five subjective synonyms (type locality in parentheses): *S. antarcticum* (Antarctica), *S. punctatum* (Antarctica), *S. roseus* (Norway), *S. sanguineum* (southern France), and *S. variabile* (Italy). Lang (1884) suggested that the slight morphological differences observed in the arrangement of the eyespots of *S. ellipse*, *S. roseum*, and *S. variabile* possibly represent a variation within a single species. Bock (1913) synonymized *S. roseum* and *S. variabile* with *S. ellipse* based on specimens collected in Sweden, Norway, South America, and South Africa. Bock (1913) also remarked that both *S. punctatum*, considered identical to *S. antarcticum*, and *S. sanguineum* possibly represent local varieties of *S. ellipse*. Likewise, Faubel (1984) considered *S. roseum*, *S. sanguineum*, and *S. variabile* as synonyms of *S. ellipse*, and uncertainly included *S. antarcticum* and *S. punctatum* in this species synonymy.

## Discussion

This study represents the first record of *Stylostomum ellipse* from the Pacific Ocean. This species shows a cosmopolitan distribution, so far recorded from the Atlantic coasts of Europe (including the Mediterranean Sea), South America, South Africa, and the Antarctic Sea (Fig. 1A). Our new findings of *S. ellipse* from the Pacific

coast of northern Japan (Fig. 1A), considered as a sub boreal zone, is in agreement with the known distribution of the species, mostly recorded from cold-temperate provinces (Holleman 2001).

While our specimens are morphologically identifiable as *S. ellipse*, there remains a possibility that this species represents two or more cryptic species, as our genetic data suggest. The 28S rDNA uncorrected *p*-distance between Hokkaido and Punat, 0.02160, was much greater than a maximum intraspecific distance, 0.00543, observed within the Caribbean population of the pseudocerotid species *Pseudoceros bicolor* Verrill, 1901 (Litvaitis et al. 2010). In addition, the partial 28S rDNA sequences of the two distinct geographical populations (South Florida vs. the Great Barrier Reef) of *P. splendidus* (Lang, 1884) have been reported to be identical (Litvaitis et al. 2019), even though the two distributions are over 10,000 km far apart. It suggests that the intra-specific genetic variation in a cotylean cosmopolitan species can be as limited as in an endemic species. Without the morphological data of the Punat material (Dittmann et al. 2019) and the molecular data from the type locality (St. Andrews, Scotland), we cannot exclude the possibility that the Punat specimen may not represent *S. ellipse* sensu stricto. Further studies are necessary to firmly establish the taxonomic identity of *S. ellipse*,



**Figure 4.** *Stylostomum ellipse*, sagittal sections, head to the left, ICHUM 6003. **A.** Diagrammatic reconstruction of copulatory complex. **B** Common pore including mouth and male gonopore. **C** Prostatic vesicle and seminal vesicle. **D** Female reproductive organ. **E** Sucker. Abbreviations: br, brain; cg, cement gland; cop, common pore; cp, cement pouch; ed, ejaculatory duct; fa, female atrium; fg, female gonopore; it, intestine; ma, male atrium; ph, pharynx; pv, prostatic vesicle; spv, spermiducal vesicle; st, stylet; sv, seminal vesicle; su, sucker; vg, vagina. Scale bars: 500  $\mu$ m (**A**); 300  $\mu$ m (**B–E**).

including a careful morphological and molecular comparison among the populations around the northeastern and south Atlantic, and from the type locality. Likewise, confirmation of the validity pertaining to the five potentially subjective synonyms *S. antarcticum*, *S. punctatum*, *S. roseum*, *S. sanguineum*, and *S. variabile* requires genetic and morphological examination of material from each type locality.

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## Authors' Contributions

YO collected samples. AT and YO conducted the histological examinations. AT performed the molecular experiments and drafted the manuscript. HK contributed to improvement of the manuscript. All authors read and approved the final manuscript.

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